APols (3, 4), a particularly successful one is folding MPs from an unfolded or denatured state to a functional one, or expressing them in vitro (5-9). Not only are these approaches of great practical interest, but they also provide a stringent test of how ''membrane-mimetic'' an environment must be in order to allow MPs to reach their native state. Their applications and implications will be discussed.

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999-Symp

New Insights into the Molecular Mechanism of Beta Barrel Outer Membrane Protein Folding

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Inspired by the seminal work of Anfinsen, investigations of the folding of small, water-soluble proteins have culminated in detailed insights into how these molecules attain and stabilise their native folds. In contrast, despite their overwhelming importance in biology, progress in understanding the folding and stability of membrane proteins remains relatively limited. Focusing on the β-barrel outer membrane protein, PagP, we have been using mutational analysis to determine how this protein folds from its urea denatured state into lipid vesicles and how this process is facilitated by molecular chaperones. In this lecture I will describe our recent experiments that have investigated the initial interactions of PagP with a bilayer, its mechanism of insertion into lipid, and how this process is facilitated by the molecular chaperones skp and surA. The work is at an early stage compared with the plethora of knowledge about the folding of water soluble proteins and how this is assisted by chaperones. Nonetheless the folding of this membrane protein is revealing new insights, new challenges and fascinating synergies with the folding mechanisms of water soluble counterparts.

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1000-Symp

Periplasmic Chaperones and their Function in the Folding of Outer Membrane Protein A into Lipid Membranes

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In Gram-negative bacteria, outer membrane proteins (OMPs) are translocated across the cytoplasmic membrane in unfolded form via the Sec translocon. Prior to insertion and folding into the outer membrane, the OMPs have to traverse the periplasm. Molecular chaperones prevent OMP aggregation before the OMPs reach the outer membrane. OMPs like outer membrane protein A (OmpA) have been shown to interact with chaperones, like Skp (1-3) and SurA (4-6).

OmpA folds spontaneously into lipid bilayers from a urea-unfolded form when the denaturant urea is strongly diluted. We have examined the kinetics of folding of OmpA and how it is affected by the chaperones Skp, SurA, FkpA. In all experiments, either in the absence or in the presence of any of these chaperones, the kinetics are well-described by two parallel kinetic processes of OmpA folding and insertion into lipid bilayers. Both processes were of first order. All examined chaperones increased the contribution of the faster folding process. However, only SurA caused an increase of the rate constant of the fast folding process. The temperature dependence of OmpA folding into lipid bilayers indicated that SurA lowers the activation energy of folding for the faster process.

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Symposium: The Synapse

1001-Symp

Reconstituting Basic Steps of Synaptic Vesicle Fusion

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Neurotransmitter release depends critically on: the SNAREs syntaxin-1, synaptobrevin and SNAP-25, which form SNARE complexes that bridge the vesicle and plasma membranes; NSF/SNAPs, which disassemble SNARE complexes; Munc18-1, which binds to syntaxin-1 and, together with Munc13, orchestrates SNARE-complex assembly; and the Ca^{2+} sensor synaptotagmin-1. Previous attempts to reconstitute neurotransmitter release revealed efficient fusion of syntaxin-1/SNAP-25-liposomes with synaptobrevin-liposomes in the presence synaptotagmin-1/Ca^{2+}, in stark contrast with physiological data showing that Munc18-1 and Munc13 are essential for neurotransmitter release. We now solve this paradox, showing that Munc18-1 displaces SNAP-25 from syntaxin-1 and that syntaxin-1/Munc18-1-liposomes fuse efficiently with synaptobrevin-liposomes in a manner that requires SNAP-25, Munc13-1 and synaptotagmin-1/Ca^{2+}. Moreover, when starting with syntaxin-1/SNAP-25-liposomes, NSF/a-SNAP disassemble the syntaxin-1/SNAP-25 heterodimers, thus inhibiting fusion, and fusion then requires Munc18-1 and Munc13-1. These results suggest that, for the first time, our experiments reconstitute synaptic vesicle fusion with the eight major components of the release machinery. We propose a model whereby the pathway to synaptic vesicle fusion does not proceed through syntaxin-1/SNAP-25 heterodimers and starts at the syntaxin-1/Munc18-1 complex; Munc18-1 and Munc13 then orchestrate membrane fusion together with the SNAREs, synaptotagmin-1 and Ca^{2+} in a manner that is not inhibited by NSF/SNAPs.

1002-Symp

Reconstitution of Calcium-Triggered Synaptic Vesicle Fusion

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The highly conserved SNARE protein family mediates membrane fusion in eukaryotic cells. We recently developed an assay to study calcium triggered synaptic vesicle fusion using single vesicle-vesicle optical microscopy. Prior to calcium injection, the system starts from a metastable state of single interacting pairs of donor and acceptor vesicles. Upon calcium injection, the system monitors content mixing (exchange or release of content) as well as lipid mixing (exchange of membrane components). Our system differentiates between vesicle docking, hemifusion, and complete fusion. Events are monitored on a hundred-millisecond time scale. We found that our system with reconstituted neuronal SNAREs, synaptotagmin-1, and complexin qualitatively mimics effects of calcium-triggered fast synchronous release. New insights into the mechanism of action of calcium-triggered synaptic vesicle fusion will be discussed.

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1003-Symp

Ultra-High Resolution Imaging Reveals Formation and Preponderance of Neuronal SNARE/Munc18 Complexes In Situ

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Membrane fusion is mediated by complexes formed by SNAP-receptor (SNARE) and Sec1/Munc18-like (SM) proteins, but it is unclear when and how these complexes assemble inside the cell. Here we describe an improved fluorescence nanoscopy technique that can achieve effective resolutions of up to 7.5 nm FWHM (3.2 nm localization precision), limited only by stochastic

photon emission from single molecules. This technique enabled us to dissect the spatial relationships between the neuronal SM protein Munc18-1 and the SNARE proteins syntaxin-1 and SNAP-25 at nanometer scales. Strikingly, we observed syntaxin-1 and SNAP-25 extensively associating with Munc18-1. Rescue experiments with syntaxin-1 mutants revealed that Munc18-1 recruitment to the plasma membrane depends on the Munc18-1 binding the N-terminal peptide of syntaxin-1, and occurs through interactions with the open conformation of syntaxin-1 that is permissive to SNARE-complex assembly. Our results, corroborated by biochemical and physiology experiments, provide unexpected insights into the functional association of SM and SNARE proteins on the neuronal plasma membrane, suggesting a general mechanism by which recruitment of an SM protein to an on-pathway SM-SNARE tri-partite complex sets the stage for membrane fusion reactions. Our high-resolution imaging approach additionally provides a novel framework for investigating interactions between the fusion machinery and other sub-cellular systems in situ.

1004-Symp
Contribution of Potassium Channels and Calcium-Activated Chloride Channels to Neuronal Signaling
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In a long-term collaboration with Yuh Nung Jan, we have taken the approach of starting our ion channel studies with molecular identification so that we can study one type of ion channel at a time. Recent studies of the contribution of potassium channels and calcium-activated chloride channels to neuronal signaling will be presented.

Platform: Voltage-gated K Channels: Gating

1005-Plat
Voltage-Dependent Gating in MthK K^+ Channels occurs at the Selectivity Filter
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The determination of how ion channels open and close is crucial for understanding their physiological roles and for potential therapeutic modulation, but locating the gate within channels is not trivial. For voltage-gated Kv channels, a primary activation-gate formed by a helical bundle crossing near the intracellular pore entrance has been established. For several types of ligand-gated channels (CNG) and large-conductance Ca^{2+}-activated K^+ (BK) channels however, the K^+ selectivity filter has been proposed to act as the conduction gate. We investigated the location of the voltage-dependent gate for the prokaryotic MthK channel, a BK channel homologue lacking voltage-sensor domains, using quaternary ammonium (QA) blockers as state-dependent probes. Intracellular QA blockers bind within an aqueous pore cavity between the putative intracellular-facing gate and the selectivity filter. Thus, these blockers ideally probe the gate location: an intracellular gate will only allow binding when open, whereas a selectivity filter gate will always allow binding. A kinetic analysis of tetrabutylammonium block of single MthK channels during gating determined that the voltage-dependent gate is located above the QA binding site in the pore. X-ray crystallographic analysis of the MthK pore with tetrabutylantimony confirmed QA binding immediately below the selectivity filter, unequivocally placing the voltage-dependent gate within the selectivity filter, akin to the C-type inactivation gate in eukaryotic K^+ channels. In addition, state-dependent binding kinetics suggested that the selectivity filter inactivation led to conformational changes within the cavity and intracellular pore entrance, suggesting an allosteric connection between cytoplasmic domains and selectivity filter.

1006-Plat
Voltage Sensor Trapping in the Relaxed State
Shobana Sundaram, **Carlos A. Villalba-Galea**.
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The voltage sensitive phosphatase from Ciona intestinalis (Ci-VSP) has constituted an intriguing model for the study of the dynamic of voltage sensing domains (VSD). Four of the five arginines in the fourth (S4) segment of the VSD function as charges sensing the difference in electrical potential across the membrane. The voltage-driven movement of the S4 segment towards the extracellular space triggers relaxation which is characterized by a shift in voltage dependence for the movement of charges to more negative values. The mechanism for relaxation remains unclear. However, it is thought to encompass the rearrangement of the VSD to satisfy the new position of the S4 segment following activation. In this view, changing the membrane potential from negative to positive voltages drives the VSD from the resting to the active state. As the S4 segment moves, the VSD gains potential energy, part of which is dissipated during a voltage-independent transition leading the VSD to the relaxed state. Replacement of the fourth arginine to a histidine (R232H) causes the VSD of Ci-VSP to display a ''pump-like'' behavior, which differs from the ''transporter-like'' behavior observed in the mutant R371H of Shaker. The cycling of this ''pump'' is driven by relaxation. Furthermore, we found that the net sensing charge of the mutant R232H reversibly seemingly decreases over 60% during this process. We concluded that after relaxation, the histidine in position 232 is deprotonated and ''trapped'' within the VSD without net charge. Similar observations are made using Molecular Dynamics simulation of the Ci-VSP voltage sensor bearing the mutation R232H. We propose electrically driving the VSD back to the resting state is inefficient after neutralizing the histidine R232H. Thus, recovery from relaxation last several seconds, diverging in two orders of magnitude the recovery observed with the native arginine.

1007-Plat
Structural Mechanism of Voltage-Dependent Gating in an Isolated Voltage-Sensing Domain
Qufei Li[1], Sherry Wanderling[1], Marcin Paduch[1], David Medovoy[1], Carlos Villalba-Galea[2], Raymond Hulse[1], Benoit Roux[1], Anthony Kossiakoff[1], Eduardo Perozo[1].
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The voltage-sensing domain (VSD) is a common scaffold responsible for the transduction of transmembrane electric fields into protein motion. They play an essential role in the generation and propagation of cellular signals driven by voltage gated ion channels, voltage sensitive enzymes and proton channels. All available VSD structures are thought to represent the activated conformation of the sensor due to the overall structural similarities and the mid-point of the voltage dependence of activation curves. Yet, in the absence of a resting state structure, the mechanistic details of voltage sensing remain controversial. The voltage dependence of the VSD from Ci-VSP (Ci-VSD) is dramatically right shifted, so that at 0 mV it presumably populates the putative resting state. We have determined crystal structures of the Ci-VSP voltage sensor in both active (Up) and resting (Down) conformations, between which the S4 undergoes a ~5 Å displacement along its main axis with an accompanying 55-90° rotation resembling the basic helix-screw mechanism of gating. In the process, the gating charges change position relative to a ''hydrophobic gasket'' that electrically separates intra and extracellular compartments. This movement is stabilized by an exchange in countercharge partners in helices S1 and S3, for an estimated net charge movement of ~1 e_o. EPR spectroscopic measurements confirm the limited nature of S4 movement in a membrane environment. These results provide an explicit mechanism of voltage sensing in diverse voltage dependent cellular responses.

1008-Plat
Tracking S4 Movements by Metal-Ion Bridges
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Electrical signalling in excitable cells depends on voltage-gated ion channels which open and close in response to changes in membrane potential. The voltage-gated ion channel consists of a central ion-conducting pore domain surrounded by four voltage-sensor domains (VSDs). The VSDs sense changes in membrane potential and confer this information to the pore domain. The fourth segment (S4) of each VSD carries several positively charged residues which gives the VSD its gating ability. S4 must traverse outwards through the membrane electric field in order for the channel to open. The open-state structures of both K and Na channels are known at atomic level through x-ray crystallography. In a previous investigation (Henrion et al., 2012, PNAS 109:8552-8557) we described four closed molecular configurations of a VSD based on 20 engineered metal-ion bridges, Rosetta modelling and molecular dynamics. A subset of these interactions was used to generate a detailed model of the intermediate conformations during VSD gating. Our results suggested that S4 slides >12 Å along its axis during gating. Whether or not S4 continues to move after channel opening and during inactivation is not clear. Therefore, in the present study, we used the same technique, with double cysteine mutations and Cd ions, to explored molecular rearrangements in the activated state. Mutated Shaker K channels were expressed in Xenopus oocytes and studied by the two-electrode voltage-clamp technique. We found several new metal ion bridges suggesting possible S4 movements in the activated state.